Bacterial-Induced Systemic Tolerance in Soybean (*Glycine max* L. Merrill) under Abiotic Stress

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SYNOPSIS

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Introduction

Abiotic stress is the principal cause of crop yield loss worldwide, reducing normal yields of major food and non food crops by more than 50 percent and thereby causing enormous economic loss as well. Drought, heat, cold, flood and salinity are among the major abiotic stresses that adversely affect plant growth and productivity. Water availability and Soil salinity are among the important abiotic factors. Water stress in its broadest sense holds both drought and flooding stress. Salinity usually accompanies water stress and may occur concurrently. Drought and salinity are becoming particularly widespread in many regions, and may cause serious salinization of more than 50% of all arable lands by the year 2050. In general, abiotic stress often causes a series of morphological, physiological, biochemical and molecular changes that unfavorably affect plant growth, development and productivity (Shanker et al. 2011).

Drought and salinity are often interrelated; these conditions induce cellular damage in singularly or combination. These stresses directly changes in the synthesis of macromolecules, and low osmotic potential in the cellular settings. In addition, these conditions lead to oxidative stress and involve the formation of reactive oxygen species (ROS) in plant cells. Under these stresses, plants have evolved complex mechanisms in order to cope with the changes taking place in their environment. The mechanisms include osmotic adjustment by accumulation of compatible solutes (Proline, sugar *etc.*), employing antioxidants and antioxidant enzymes (SOD, POX, CAT *etc.*) for effective scavenging of reactive oxygen species and lowering the toxic concentrations of ions in the cytoplasm by restriction of Na^+ influx or its sequestration into vacuole and/or its extrusion (Mahajan et al. 2005). In addition, plants induce signaling cascades that activate ion channels, kinase cascades (CDPKs, MAPKs) and several transcription factors (Cheng et al. 2002). Phytohormones also play central roles in abiotic and biotic stress signaling. SA, JA and ET have central roles in biotic stress signaling. ABA is involved in the response to abiotic stress as low temperature drought and osmotic stress (Crozier et al. 2000).

The variation between salt tolerant (halophytes) and salt sensitive (glycophtes) genotypes provide a genetic basis for engineering salt-tolerant crops. Strategies for making tolerant plants to salinity and produce economically valuable species have been extensively studied for decades. A variety of strategies have been used to improve the tolerance of crops, including traditionally (breeding), chemically (priming agents), molecular method (genetic engineering) and biologically (biofertilizers). Plant growth promoting rhizobacteria (PGPR) as inoculums in

agriculture to alleviate salt stress is the most promising approach to enhance production and yield in salinity-affected regions. In the last decade, bacteria belonging to different genera including *Rhizobium, Bacillus, Pseudomonas, Pantoea, Paenibacillus, Burkholderia, Achromobacter, Azospirillum, Microbacterium, Methylobacterium, Variovorax, and Enterobacter* etc. have been reported to provide tolerance to host plants under different abiotic stress environments (Choudhary et al. 2012). PGPRs are a group of bacteria that can actively colonize the rhizosphere or endorhizosphere of plants and promote growth of the plants through various direct and indirect mechanisms. Indirect mechanisms have been observed with most PGPR strains by reducing plant pathogens. In this mechanism, bacteria secrete antibiotics and lytic enzymes that provide resistance against pathogen attack. Direct mechanisms may act on plant itself and affect growth by facilitating the nutrient uptake through phytohormone production, lowering of plant ethylene levels, production of siderophores, solubilization of minerals and fixation of atmospheric nitrogen. This results in 'Induced Systemic Tolerance (IST)' which is an important process to overcome the harmful effects of abiotic stress. It is PGPR induced physical and chemical changes in plants to alleviate environmental stress.

Soybean is a species of legume widely grown for its edible bean, providing oil and protein. Together, soybean oil and protein content account for about 60% of dry soybeans by weight (protein at 40% and oil at 20%). The main soybean producing states in India are Madhya Pradesh (53 percent), Maharashtra (34 percent), and Rajasthan (8 percent). Rajasthan is the third largest soybean producing state (USDA report, 2013). The soybean producing regions in Rajasthan are mainly affected by high temperature, soil salinity/alkalinity, low pH and metal toxicity (DST-Rajasthan report). Soybean is incapable of growing in drought and salt stresses as it may be devoid of mechanisms to survive in stressed conditions or unavailability of nutrient resulting in decrease the yield of soybean in Rajasthan. Use of PGPR in soybean field can result in the promotion of plant health by alleviation of such stresses and play a significant role in low-input sustainable agriculture applications.

Objectives of the study

This study mainly focuses on the induced systemic tolerance in bacterial-treated soybean plants under abiotic stress.

The objectives selected for this study are as follow:

1. Isolation of rhizobacteria and their screening on the basis of stress tolerant and plant growth promoting activity
2. Optimization and evaluation of selected bacteria and priming agents for soybean growth promotion in pot trials under salt affected soil
3. Biochemical and molecular characterization of effective PGPR
4. Elucidation the mechanisms of induced systemic tolerance in soybean plant

Review of literature

Plant growth promoting rhizobacteria are the group of bacteria that colonize the plant roots, firstly described by Kloepper and Schroth (1978). PGPR are mostly present in near the roots due to presence of root exudates secreted by plants and used as nutrient source by them. The nature of root exudates and soil conditions affect diversity of PGPR and their interaction with host plants. These bacteria are directly or indirectly involved in plant growth promotion via production and secretion of various regulatory chemicals in the vicinity of rhizosphere. The use of biological products based on PGPR inoculums in agriculture offer an environmentally sustainable approach for maintaining the healthy yield of crops in saline habitat. Various traits of PGPR have been reported on amelioration of salinity stress, e.g., ACC-deaminase, expolysaccharide, volatiles production, Pi soulbilization, IAA production etc.

Early studies on induced systemic tolerance (IST) to drought, Timmusk et al. (2000) reported that inoculation with the PGPR *Paenibacillus polymyxa* enhanced the drought tolerance of *Arabidopsis thaliana* by augmented gene expression of *ERD15* gene. Mayak et al. (2004) worked on an another bacterial strain *Achromobacter piechaudii* ARV8, which produces 1-aminocyclopropane-1-carboxylate (ACC) deaminase, conferred induced IST to drought stress in pepper (*Capsicum annuum* L.) and tomato (*Solanum lycopersicum* L.) plants. PGPR have ability to improve plant water relations by enhancing the accumulation of osmolytes in plants. *Bacillus* sp. inoculated plants root accumulated higher proline content in salt stress compared to non-inoculated plants root due to higher uptake of nutrients, resulting high biosynthesis rate (Vardharajula et al. 2011). Another mechanism has been proposed in the water stress avoidance mediated by PGPR, maintenance the root hydraulic conductance under saline environment which was strongly correlated with increased root expression of aquaporin isoforms (Marulanda et al. 2010). The contribution of PGPR towards toxic ions homeostasis is reported to improve plant growth and tolerance under salinity. Microbes can restrict the uptake of toxic ions by regulating ion transporter expression and formation of rhizosheaths by bacterial exopolysaccharides (EPS) work as a physical barrier around the roots. Ashraf et al. (2004) explained that *Aeromonas hydrophila/caviae* and *Bacillus* sp. decreased Na^+ accumulation in wheat plants by the excretion of exopolysaccharides, which bind Na^+ in roots, and preventing their transfer to leaves. The authors suggested that EPS covered a higher proportion of the root of bacterial inoculated seedlings, which reduced flow of sodium ions into the stele. Khodair et al. (2008) described that

exopolysaccharide producing bacteria have potency to restrict Na+ influx into roots, thus enabling the salt-sensitive plants to survive and flourish under salt stress. In another case of PGPR mediated decreased plant Na^+ accumulation could be explained by the exposure of bacterial volatiles (VOCs), which can modulate the Na^+ homeostasis pathway. *Arabidopsis* plants exposed to *B. subtilis* GB03 VOCs were exhibited less susceptible to salt stress than control plants. *B. subtilis* decreased root transcriptional expression of a high-affinity K^+ transporter (*AtHKT1*) but up-regulated it in the shoots facilitating root-to-root Na^+ recirculation (Shkolnik-Inbar et al. 2013).

Phosphate solubilizing bacteria (PSB) excrete low molecular weight organic acids such as gluconic acid, citric acid, succinic acid, propionic acid, lactic acids that mineralize and dissolve organic phosphate compounds and make available to plants in the form of inorganic phosphate (Choudhary, 2012). Salinity cause the depletion and precipitation of available phosphorus and PSB have been tendancy to solubilize precipitated forms of phosphorus in hydroponic MS medium and enhanced the phosphorus content in plant system under NaCl stress (Shukla et al. 2012; Vaishnav et al. 2015). PGPB moreover make the plant to grow in nitrogen deficient soil by fixing nitrogen (Dobbelaere et al. 2003). Nitrogen fixation in legume plants is mediated mainly by associative bacteria *Rhizobium*. Salinity has a major effect on legume biology as it affects the survival and distribution of rhizobia in soil and their interaction with legume plants. After understanding about the sensitivity of the host legumes to salt stress, most of the work done with co-inoculation of *Rhizobium* and PGPR. The co-inoculation of PGPR is a good strategy when *Rhizobium* is not so effective in saline environment. PGPR strains contain ACC deaminase enzyme has been observed to reduce the ethylene concentration that causes negative impact on nodulation in legumes under stress environment (Ahmad et al. 2011). *Pseudomonas* and *Rhizobium phaseoli* co-inoculation was observed very effective for enhancing nodulation process in mung bean plants under laboratory as well as field conditions affected by salinity stress (Ahmad et al. 2013). Siderophores are iron chelating compounds proved in different PGPR strains as an important attribute for plant growth promotion and phyopathogen protection (Scavino and Pedraza, 2013). PGPR secrete siderophore in the rhizosphere and then plant roots uptake iron from siderophore by either chelate degradation or direct uptake (Rajkumar et al. 2010). Production of indole acetic acid (IAA), and gibberellic acid (GA) results in increased root length, root surface area and number of root tips, leading to enhanced uptake of

nutrients thereby improving plant health under stress conditions (Patten and Glick 2002; Mantelin and Touraine 2004; Egamberdieva and Kucharova 2009). Many soil bacteria contain ACC deaminase (ACC-D) enzyme, which cleaves ACC to α-ketobutyrate and ammonia and thereby lowers the ethylene level in stressed plants. There are several reports that showed ACC-D containing PGPR can decrease salinity induced growth inhibition (Ahmed et al. 2011; Wu et al. 2012). PGPR strain *Achromobacter piechaudii* ARV8 which produced ACC-D was found to significantly increase the fresh and dry weights, uptake of P and K and water use efficiency of tomato seedlings in the presence of NaCl stress (Mayak et al. 2004).

Methodology

1. **Sample collection**

   Soil samples will be collected from Bundi district of Rajasthan and Madhya Pradesh soybean field.

2. **Isolation and screening of bacterial strain**

   Bacterial cultures will be isolate from the soybean rhizospheric soil samples by serial dilution and agar plating method. Recovered isolates will be maintained on Nutrient agar media (NA) plates and plates will be incubated in an incubator at 28 ± 2°C for 24 to 36 hours. A 5 ml mixtures of, autoclaved glycerol + Nutrient broth cultures (1:1 v/v) will be preserved in cryovials at –20°C. Bacterial isolates will be screened on the basis of NaCl & PEG stress, plant growth promotion (PGP) properties and pot trials. PGP characteristics will be performed by standard protocols; IAA production (Gordon and Weber, 1951), ACC deaminase activity (Penrose and Glick, 2003), phosphate solubilization (Pikovskaya's agar medium), siderophore production (Schwyn and Neilands, 1987) and EPS production De Vuyst et al. 1998).

3. **Molecular characterization of structural and functional genes**

   Promising strains will be selected and identified by using 16S rRNA gene sequencing technique. Different type of PGP properties genes will be amplified for functional characterization. All gene sequences will be submitted in NCBI database. Potent bacterial culture will be submitted in MTCC, IMTECH Chandigarh for open access.

4. **Screening of a potent priming agent for plant growth promotion**

   Different type of priming agents (Proline, glycinebetain, hydrogen peroxide, mannitol etc.) will be screened for soybean seed germination and plant growth promotion under salt stress conditions.

5. **Role of bacterium in IST**

   Selected strains will be analyzed *in vitro* for plant growth promoting activities in soybean plants under salt stress. Bacterial inoculum will be prepared by harvesting bacterial cells from 24h old cultures on NA plates at 26°C. The inoculum prepared in sterile physiological saline and then will be inoculated in soil or seed for potency. Different type of plant growth parameters (physiological, biochemical and molecular) will be performed. Antioxidant enzymes activity will be performed in soybean plants by

spectrophotometric analysis. The expression of stress responsive genes will be analyzed in soybean plants by qRT-PCR technique.

6. **Publication of research papers/patents and thesis writing**

Publication of research results in International and National journals, books, symposiums and conferences will be accomplished.

Findings

In an attempt to study the isolation of plant growth promoting bacteria, 43 isolates were recovered from soybean rhizospheric soil grown in Bundi district Rajasthan. All isolates were screened based on physiological and plant growth promotion properties.

- One bacterial isolate AU was found to produce IAA, ACC-D, siderophore, exopolysaccharide, Pi solubilisation and beneficial VOCs production under 10% NaCl condition.
- Further, AU isolate was selected for induced systemic tolerance study and subjected to biochemical and molecular characterization. Based on the morphological and biochemical properties, AU isolate was tentatively identified as *Pseudomonas* sp. After molecular characterization with 16S rRNA gene, AU strain showed a similarity of 99.93% with *Pseudomonas simiae* OLi type strain (AJ936933) with '*P. fluorescens* intrageneric cluster'.
- In addition, PCR amplification was confirmed the presence of ACC-deaminase, IAA production, gluconic acid, siderophore, trehalose, nitrite reductase and proline gene in bacterial isolate AU. All gene sequences of AU isolate were submitted to NCBI accession number and the culture was deposited in an IDA approved culture collection, Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh.
- The inoculation of bacterial strain AU applied in soil along with 100µM sodium nitoprusside (SNP) for 100 mM NaCl stress challenged soybean plants alleviated the salinity stress and promoted the plant growth with increased plant growth characteristic. SNP treatment was found to enhance *P. simiae* colonization efficiency with soybean plant root under 100 mM NaCl stress. Application of SNP + bacterium AU enhanced proline and IAA content, peroxidase, catalase, superoxide dismutase enzymes activity, nutrient content and distribution of sodium ions in soybean plants under salt stress.
- Plant proteins were characterized by MALDI-MS/MS followed by western blotting analysis. The expression study confirmed that vegetative storage protein (VSP), gamma-glutamyl hydrolase (GGH) and RuBisCo large chain proteins were significantly up regulated after inoculation of SNP + bacterium AU in soybean plant under salt stress. Gene expression study showed that SNP + bacterium AU treatment regulates expression of salt responsive genes. Expression of Peroxidase (*POX*), catalase (*CAT*), vegetative storage protein (*VSP*) and nitrite reductase (*NR*)

genes were up-regulated, whereas high affinity K^+ transporter (*HKT1*), lipoxygenase (*LOX*), polyphenol oxidase (*PPO*) and pyrroline-5-carboxylate synthase (*P5CS*) genes were down-regulated in soybean leaves under salt stress.

- Treatment of SNP has significantly enhanced biofilm formation and VOCs production in AU isolate. GC-MS analysis showed diversity in bacterial mediated VOCs expression under different treatments. Five volatile compounds as pure chemicals (1- hexanol, benzothiazole, tridecane, 4-nitroguaiacol and quinolone) we were tested five volatile compounds as pure chemicals (1- hexanol, benzothiazole, tridecane, 4-nitroguaiacol and quinolone) at different concentration range. Chemotaxis assay revealed that SNP treatment altered root exudates profiling which was found more attracted to AU bacterial cells as compared to non-treated root exudates under salt stress.
- From the above study it can be concluded that the promising results were observed on using bacterial strain *Pseudomonas simiae* strain AU. The strain fruitfully alleviated salt stress in soybean plants which provides a concrete support for use of this bacterium as biofertilizer for salt stress challenged plants. In addition, the application of Nitric oxide represents an attractive technology to modify the rhizosphere with potential novel applications in agriculture.

Significance of work

PGPR can be termed a biological means for plant growth promotion but knowledge about the mechanisms underlying PGPR-induced systemic tolerance is still very fragmentary. In an attempt to fill this gap, this study will be useful. The isolated bacterial strains from this study can be tested for field trials as a biofertilizer to make the soil nutrients available to plants. The introduction of salt tolerant PGPR into soybean cultivation system could be a useful low input strategy for reduce fertilization excess and improve soybean production under saline condition.

References

- Ahmad M, Zahir ZA, Asghar HN, Asghar M (2011) Inducing salt tolerance in mung bean through coinoculation with rhizobia and plant-growth-promoting rhizobacteria containing 1-aminocyclopropane-1-carboxylate deaminase. *Can J Microb* 57(7):578-89.
- Ahmad M, Zahir ZA, Khalid M, Nazli F, Arshad M (2013) Efficacy of Rhizobium and Pseudomonas strains to improve physiology, ionic balance and quality of mung bean under salt-affected conditions on farmer's fields. *Plant Physiol Biochem* 63:170-6
- Ashraf M, Berge SH, Mahmood OT (2004) Inoculating wheat seedling with exopolysaccharides-producing bacteria restrict sodium uptake and stimulates plant growth under salt stress. *Biol Fertil Soils* 40:157-162
- Cheng S-H, Willmann MR, Chen H-C, Sheen J (2002) Calcium Signaling through Protein Kinases. The *Arabidopsis* Calcium Dependent Protein Kinase Gene Family. *Plant Physiol.* 129: 469-485
- Choudhary DK (2012) Microbial rescue to plant under habitat imposed abiotic and biotic stresses. *Appl Microbiol Biotechnol* 96:1137 1155
- Crozier A, Kamiya Y, Bishop G, Yokota T (2000) Biosynthesis of Hormones and Elicitor Molecules. *In* BB Buchanan, W Gruissem, RL Jones, eds, Biochemistry & Molecular Biology of Plants. *American Society of Plant Biologist* Rockville, Maryland. U.S.A., pp 850-929
- De Vuyst L, Vanderveken F, Van de Ven S, Degeest B (1998) Production by and isolation of exopolysaccharides from *Streptococcus thermophilus* grown in milk medium and evidence for their growth-associated biosynthesis. *J Appl Microbiol* 84:1059-68
- Gordon SA, Weber RP (1951) Colorimetric estimation of indole acetic acid. *Plant Physiol* 26: 192-195
- Khodair TA, Galal GF, El-Tayeb TS. Effect of inoculating wheat seedlings with exopolysaccharide-producing bacteria in saline soil. *J. Appl. Sci Res.* 2008;4:2065-70.
- Khodair, T.A., Gehan F. Galal and T.S. El-Tayeb (2008) Effect of inoculating wheat seedlings with exopolysaccharide-producing bacteria in saline soil. *J Appl Sci Res* 4(12): 2065-2070

- Kloepper JW, Schroth MN (1978) Plant growth promoting rhizobacteria on radishes. In: IV 647 International conference on plant pathogenic bacteria, vol 2. Station de Pathologie Vegetale 648 et Phytobacteriologie, INRA, Angers, France, pp 879–882
- Mahajan S., Tuteja N. (2005) Cold, salinity and drought stresses: An overview. *Arch Biochem Biophysics* 444:139–158
- Marulanda A, Azco´n R, Chaumont F, Ruiz-Lozano JM, Aroca R (2010) Regulation of plasma membrane aquaporins by inoculation with a *Bacillus megaterium* strain in maize (*Zea mays* L.) plants under unstressed and salt-stressed conditions. *Planta* 232: 533–543
- Mayak, S. et al. (2004) Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. *Plant Physiol. Biochem.* 42, 565–572
- Penrose DM, Glick BR (2003) Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. *Physiol Plant* 118: 10-15
- Rajkumar M, Ae N, Prasad MN, Freitas H (2010) Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. *Trends Biotechnol* 28(3):142-9.
- Scavino AF, Pedraza RO (2013) The role of siderophores in plant growth-promoting bacteria. D.K. Maheshwari et al. (eds.), Bacteria in Agrobiology: Crop Productivity. DOI 10.1007/978-3-642-37241-4_11
- Schwyn B, Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem* 160:47–56
- Shanker K.A and B. Venkateswarlu (2011) Abiotic Stress Response in Plants – Physiological, Biochemical and Genetic Perspectives, ISBN 979-953-307-195-3
- Shkolnik-Inbar D, Adler G, Bar-Zvi D (2013) ABI4 downregulates expression of the sodium transporter *HKT1* in *Arabidopsis* roots and affects salt tolerance. *Plant J* 73: 993–1005
- Shukla PS, Agarwal PK, Jha B (2012) Improved salinity tolerance of *Arachis hypogaea* (L.) by the interaction of halotolerant plant growth promoting rhizobacteria. *J Plant Growth Regul* 31: 195–206
- Timmusk, S. and Wagner, G.H. (1999) the plant-growth-promoting rhizobacterium *Paenibacillus polymyxa* induces changes in *Arabidopsis* thaliana gene expression: a

possible connection between biotic and abiotic stress responses. *Mol. Plant Microbe Interact.* 12, 951–959

- Vaishnav A, Kumari S, Jain S, Varma A, Choudhary DK (2015) Putative bacterial volatile-mediated growth in soybean (*Glycine max* L. Merrill) and expression of induced proteins under salt stress. *J Appl Microbiol* 119: 539-551.
- Vardharajula S, Ali SA, Grover M, Reddy G, Bandi V (2011) Drought-tolerant plant growth promoting *Bacillus* spp.: effect on growth, osmolytes, and antioxidant status of maize under drought stress. *J Plant In* 6: 1-14
- Wu XX, Zhu WM, Zhang H et al (2011) Exogenous nitric oxide protects against salt-induced oxidative stress in the leaves from two genotypes of tomato (*Lycopersicom esculentum* Mill.). *Acta Physiol Plant* 33: 1199–1209